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Sir:

We submit herewith the certified copy of European Patent Application No. 01402406.1, filed September 19, 2001, the priority of which is hereby claimed.

Respectfully submitted,

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Bescheinigung

Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.

Certificate

The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Attestation

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécificée à la page suivante.

Patentanmeldung Nr. | **Patent application No.** | **Demande de brevet n°**

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The organization code and number of your priority application, to be used for filing abroad under the Paris Convention, is EP01402406

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le President de l'Office européen des brevets
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Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:
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Molecules binding to glu-pro motifs, therapeutical compositions containing them and their applications

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MOLECULES BINDING TO GLU-PRO MOTIFS, THERAPEUTICAL COMPOSITIONS CONTAINING THEM AND THEIR APPLICATIONS.

The present invention relates to molecules binding to specific targets comprising Glu-Pro (EP) repeated motifs such, for example the lymphocyte activation gene-3 (lag-3)-associated protein hereafter named LAP . The invention relates too, to therapeutical compositions containing said molecules, to antibodies directed against said molecules, to therapeutical compositions containing them . Also, the invention relates to methods for screening drugs useful for the treatment of immune disorders.

In previous work, the applicant showed that both LAG-3 and MHC class II were present in the cell fraction of glycosphingolipid-rich complexes (GSL complexes) before the assembly of the immunological synapse by CD3/TCR complex crosslinking.

Using the LAG-3 intracytoplasmic region as bait in the yeast two-hybrid cloning system, applicant have now identified a novel interaction between a new human protein termed LAP for LAG-3-Associated Protein and EP repeated motifs present in LAG-3.

The applicant shows that LAP binds specifically *in vitro* and *in vivo* to the Glu-Pro (EP) repeated motif present in the LAG-3 intracytoplasmic region and that LAP also binds to the EP motif of another functionally important receptor, the PDGFR.

The applicant shows that such an interaction plays an important role in T cell function and homeostasis

because LAG-3 acts as a negative regulator of activated T-cells and plays an important role in regulating the expansion of activated T-cells and limiting antigen induced cell death.

LAG-3 associates with the TCR:CD3 complex and interferes with TCR signalling. This down regulation may be activated by disrupting CD4 and CD8 co-receptor function since LAG-3 is expressed on both $CD4^+$ and $CD8^+$ cells and has been shown to be associated with CD4 and CD4 in raft microdomains.

The LAP protein is likely to transduce the appropriate signals that lead to this control on T cell function and CD4 and CD8 T cell subpopulation homeostasis.

This negative control on T cell activation is of prime importance for the regulation of primary activated T-cells as well as the regulation of T-cell memory development and homeostasis.

LAP protein is encoded by a 1.8 kb RNA message in lymphocytes that is derived from a rare mRNA and encodes a 45 kDa protein that is expressed in most tissues.

Thus, molecules that, as LAP, bind to the EP motif are candidates molecules for a new type of signal transduction and/or coupling of clustered rafts to the microtubule networks that could explain how negative signalling of co-receptors may occur through molecules devoid of any immunoreceptor tyrosine-based inhibitory motifs (ITIM) consensus sequence.

The work conducted by the applicant allow him to verify that supramolecular assemblies between LAG-3, CD3, CD8 and MHC class II molecules result from the

organization within raft microdomains (Hannier, S. and Triebel, F., *The MHC class II ligand LAG-3 is co-distributed with CD8 and CD3/TCR molecules after their engagement by mAbs or peptide/MHC class I complexes,* Int. Immunol. 1999. 11: 1745-1752).

To investigate the pathway involved in LAG-3-dependent TCR signalling regulation, applicant directly clone proteins expressed in activated T cells that would specifically bind to the IC region of hLAG-3.

Using the yeast two-hybrid system and the LAG-3 IC region as bait, the applicant identified a novel protein, termed LAP for LAG-3-associated protein that binds to the Glu-Pro (EP) repeated motifs present within the LAG-3 IC region C-terminus.

These Glu-Pro (EP) repeated motif are present, for example, in the LAG-3 intracytoplasmic region and in the functionally important receptor named Platelet Derived Growth Factor Receptor (PDGFR). Other intracellular signalling molecules including this unusual EP motif are SPY75 and lckBP1, the mouse homologues of the human HS1 product. These molecules have been shown to be involved in TCR signalling.

Then, the present invention relates to molecules binding to a target comprising an EP motif, in particular, to molecules binding to a target comprising an EP motif having the following sequence :

$$[X-(EP)_n-Y-(EP)_m-Z]_p$$

wherein X, Y and Z, identical or different comprise a sequence of 0 to 10 aminoacids, identical or different, n, m are integers comprised between 0 to 20,

preferably between 3 to 10 at least one from n or m being different from 0 and p is an integer comprised between 1 and 10.

In a particular embodiment the invention relates to a molecule which binds to an EP motif selected from the group comprising the following formula: EPEPEPEPEPEPEPEPEP (SEQ ID N° 3), EPEPEPQLEPEP (SEQ ID N° 4), EPQDEPPEPQLELQVEPEPELEQ (SEQ ID N° 5), or EPEPEPEPEPEP (SEQ ID N° 6).

In another particular embodiment the invention relates to a molecule that binds to an aminoacid sequence comprising at least 5 EP motifs over a 19 aminoacid length segment.

The molecule of the invention is selected from a peptide, a polypeptide or a protein.

Preferably the molecule of the invention is a purified polypeptide consisting of or comprising the amino acids sequence identified by SEQ ID No.:1 an homologous, a fragment or a derivative thereof.

More preferably the molecule of the invention is a purified polypeptide consisting of or comprising the carboxy-terminal amino acids sequence of LAP identified by SEQ ID No.:2 of the sequence listing in annexe, an homologous, a fragment or a derivative thereof.

For the purpose of the present invention:

- an homologous polypeptide relates to a polypeptide or a which can differ by one or a few amino acid residues when compared with the polypeptide of the invention, as the polypeptides identified by SEQ ID No. :1 or SEQ ID No. :2, but that maintain all the biological

functions of said polypeptide, namely, his capacity to bind glu-pro motifs.

- a polypeptide fragment relates to any amino acid sequence contained in the sequence of the polypeptide of the invention, which maintains the binding capacity for at Glu-Pro motifs,

- a polypeptide derivative relates to said entire or fragment polypeptides, labelled with chemical or biological entities in order to be easily detected. Chemical or biological entities may be enzymes, fluorescent labels, coloured particles, etc.

The invention also relates to a nucleic acid molecule consisting of or comprising a polynucleotide sequence coding a polypeptide according to the invention and particularly to a nucleic acid molecule coding for the polypeptide identified by SEQ ID No. : 1 from the sequence listing in annexe.

Also the invention relates to a nucleic acid molecule, consisting of or comprising the polynucleotide sequence identified by SEQ ID No.:8 a fragment or a derivative thereof.

The invention relates also to an expression vector comprising a nucleic acid molecule according to invention.

For the purpose of the present invention, an "expression vector" refers to any replicable DNA construct used either to amplify or to express DNA, which encodes one of the polypeptides of the invention.

The invention also relates to a host cell transformed with an expression vector according to invention.

Host cells may be prokaryotic or eukaryotic, including but not limited to bacteria, yeasts, insect cells, mammalian cells, including cell lines, which are commercially available.

The invention is also directed to a process for the manufacturing of a purified polypeptide according to the invention, comprising :

a) the transfection of a host cell with an expression vector according to the invention to obtain the expression of the polypeptide,

b) the isolation and purification of the polypeptide from the transfected host cell.

Purification of said polypeptide may be accomplished by any standard methods for purification of membrane or soluble proteins

The invention is also relating to a pharmaceutical composition comprising as active agent at least one molecule according to the invention.

The pharmaceutical compositions of the invention are useful for treating immune-related pathologies and in particular they are useful for modulating the immune response.

In a particular embodiment, the pharmaceutical compositions of the invention are useful to enhance the development of CD4 or CD8 T-cell populations.

In another particular embodiment, the pharmaceutical compositions of the present invention are also useful to suppress the development of CD4 or CD8 T-cell populations.

The pharmaceutical composition of the invention comprise as active agent a LAP agonist.

In another particular embodiment, the pharmaceutical composition of the invention comprise as active agent a LAP antagonist.

For the purpose of the present invention a LAP agonist is any molecule that mimics the effect of LAP binding when it binds to the target EP motifs and a LAP antagonist is any molecule that inhibits the effect of LAP binding when it binds to the target EP motif.

The invention also include the use of a molecule according to invention for the manufacture of a pharmaceutical composition useful for treating immune-related pathologies or for modulating the immune response

The invention relates to the use of a molecule according to invention for the manufacture of a pharmaceutical composition enhancing the development of CD4 or CD8 T-cell populations.

The invention relates to the use of a molecule according to invention for the manufacture of a pharmaceutical composition suppressing the development of CD4 or CD8 T-cell populations

In a particular embodiment, the molecule used

to prepare pharmaceuticals compositions according to invention is a LAP agonist.

In a particular embodiment, the molecule used to prepare pharmaceuticals compositions according to invention is a LAP antagonist

The invention also includes a method for screening drugs comprising the steps of :

-put in contact the drug candidate with a molecule according to the invention in the presence of her target EP motif,

-measure the resulting binding of said molecule to her target.

The method for screening drugs according to the invention allows the screening of drugs selected from the group comprising drugs able to activate T-cell, drugs enhancing the development of CD4 or CD8 T-cell populations, drugs suppressing the development of CD4 or CD8 T-cell populations, drugs active in platelet activation

Preferably the molecule according to the invention to put in contact with the drug candidate in the screening method is a LAP polypeptide.

The invention also relates to antibodies directed to a specific epitope of the polypeptide identified by SEQ ID NO:1.

In particular embodiments, antibodies according to invention are monoclonal antibodies or polyclonal antibodies or Fab, Fab', F(ab') or Fv fragments thereof.

The scope of the invention also comprises a monoclonal or polyclonal antibody or a monoclonal or polyclonal antibody fragments or derivatives which specifically binds a peptide of SEQ ID NO:1, said monoclonal or polyclonal antibody derivative being selected from the group consisting of a monoclonal or polyclonal antibody conjugated to a cytotoxic agent or a radioisotope, and Fab, Fab' or $F(ab')_2$ fragments of said monoclonal or polyclonal antibody conjugated to a cytotoxic agent or radioisotope.

Antibody fragments are regions from said polyclonal or monoclonal antibodies sequences recognising at least one epitope present in the peptide of SEQ ID NO:1, which maintain the binding capacity for at least one of, said epitopes.

Antibody derivatives are entire or fragment antibodies labelled with chemical or biological entities in order to be easily detected. Chemical or biological entities may be enzymes, fluorescent labels, coloured particles, etc.

The invention relates also to a hybridoma cell line producing a monoclonal antibody according to the invention.

The present invention directs also to a therapeutic composition comprising as active ingredient an antibody according to the invention.

The present invention is also relating to the use of said antibodies in a method for purifying, identifying or quantifying a polypeptide defined in claim 1 or its homologous.

The present invention is also relating to the use of said antibodies to screen compounds active in intracellular signalling mediated by cell surface receptor.

The present invention is also relating to the use of said antibodies to screen compounds active in T-cell activation or the regulation of the expansion of activated T-cells.

The present invention is also relating to the use of said antibodies to screen compounds active in platelet activation.

The present invention is also relating to the use of said antibodies for the manufacture of a therapeutic composition useful for treating immune-related pathologies.

The present invention is also relating to the use of said antibodies for the manufacture of an immunomodulatory pharmaceutical composition.

The present invention will be comprised in lecture of following experimental results and in figures in annexe where:

Figure 1 represents the *in vitro* interaction of human LAP with hLAG-3.

Figure 1A shows that LAP binds specifically to the natural hLAG-3 (70 kDa) protein present in whole cell lysate of PHA-activated human PBMCs

Figure 1B shows that LAP binds specifically to a protein produced by in vitro translation of an hLAG-3 mRNA in a rabbit reticulocyte lysate.

Figure 2 illustrates interactions tested in the two-hybrid system using co-transformation with two plasmids and mating of two yeast strains.

Figure 2A shows three partial LAP proteins (D1, D2 and D3) lacking their C-terminal domain were cloned in frame with the GAL4 AD protein, using a partial 1104 bp LAP cDNA.

Figure 2B shows that the EP-rich C-terminal region of the PDGF receptor (PDGFR) was fused with the LexA BD.

Figure 2C shows interactions in the two-hybrid system.

Figure 3 represents Western blots autoradiograms obtained with the anti-LAP immune serum, revealing a specific band at 45 kDa.

Western blots were performed using 10 µl total cell lysates of PBMC (lanes 2, 4, 6) or PHA blasts (lanes 1, 3, 5). The blots were incubated in rabbit preimmune serum (lanes 1, 2), rabbit polyclonal antibody against LAP (lanes 3, 4) or the latter preincubated with 10-6 M LAP peptide (lanes 5, 6). The arrow indicates the LAP 45 kDa protein.

Results and discussion:

2.1 LAG-3 and MHC class II are expressed in GSL complexes on the surface of human activated T cells

GSL complexes (raft microdomains) were isolated in a low-density fraction, at the interface between the 35% and 5% fractions of a discontinuous sucrose gradient, as described by Montixi et al. (Montixi, C., Langlet, C., Bernard, A. M., Thimonier, J., Dubois, C., Wurbel, M. A., Chauvin, J. P., Pierres, M. and He, H. T., Engagement of T

cell receptor triggers its recruitment to low-density detergent-insoluble membrane domains *The EMBO Journal* 1998. 17: 5334-5348). Twelve fractions of the gradient were analysed by Western-blotting. LAG-3, DR-α as well as p56lck were detected in fraction 9, representing the GSL complex isolates, and were not detected anymore following addition of 0.2 % saponin (cholesterol depletion leading to raft disruption) to 1 % Triton X-100 (data not shown). CD45, a phosphotyrosine phosphatase known to be excluded from raft microdomains was used as a negative control. Thus, LAG-3 is present in raft microdomains before engagement of the TCR by specific mAb or peptide/MHC complexes.

In addition, it was found that MHC class II (DR-α) molecules were also present in raft microdomains on activated T cells (results not shown). The partitioning of MHC class II into the raft fraction has been reported to occur in the myelomonocytic THP-1 cells following their crosslinking with antibodies and to be mandatory for protein tyrosine kinase (PTK) activation (Huby, R. D. J., Dearman, R. J. and Kimber, I., Intracellular phosphotyrosine induction by major histocompatibility complex class II requires co-aggregation with membrane rafts J. Biol. Chem. 1999. 274: 22591-22596). In B cells, MHC class II were found to be constitutively present in rafts and this concentration of MHC class II molecules facilitates antigen presentation (Anderson, H. A., Hiltbold, E. M. and Roche, P. A., Concentration of MHC class II molecules in lipid rafts facilitates antigen presentation Nature Immunol. 2000. 1: 156-162).

The presence of LAG-3 in raft microdomains before engagement of the TCR argues for its close

association with CD3/TCR complexes and explains, in part, previous observations where LAG-3 was found to be co-clustered with CD3/TCR complexes and also with CD8 in co-capping experiments (Hannier, S. and Triebel, F., The MHC class II ligand LAG-3 is co-distributed with CD8 and CD3/TCR molecules after their engagement by mAbs or peptide/MHC class I complexes Int. Immunol. 1999. 11: 1745-1752).

2.2 Isolation of a novel human protein, LAP, interacting with LAG-3

An interaction screening was performed by using the yeast two-hybrid system to identify proteins that bind to the intracellular domain of human LAG-3 *in vivo*. First, it was verified that no LAG-3 construct in pLex or pLex/NLS displayed any lacZ reporter gene activity in yeast cells expressing pGAD without insert. This indicated that LAG-3 does not show any non-specific binding to DNA sequences leading to GAL promoter activation. Then, strain L40 was transformed with pLex/NLS-hLAG-3/I to screen about 2 x 10^5 colonies of the human activated T-cell cDNA library. Around 200 colonies that grew on histidine-free drop-out medium were selected, replaced onto selective medium and assayed for β-galactosidase expression. From these, 13 showed reporter gene activities. In order to confirm the specificity of these interactions, the plasmid DNA from selected clones was isolated and used for transformation of the strain AMR70, which were then mated with strain L40 containing either the bait plasmid pLex/NLS-hLAG-3/I or a control plasmid (pLex-Lamin or pLex/NLS-RalB). Three specific clones were obtained showing strong interaction with hLAG-3/I (signals appeared in less than 2 hrs) and not with Lamin or RalB. The inserts of these clones were submitted to restriction mapping and sequence analysis. The

three cDNAs were found to encode a unique partial (i.e. lacking the ATG translation initiation codon) sequence of 243 amino acids, termed LAP (not shown). This novel molecule has some homology with the C terminal region of the TCP-10 protein previously cloned in human (Islam, S. D., Pilder, S. H., Decker, C. L., Cebra-Thomas, J. A. and Silver, L. M., *The human homolog of a candidate mouse t complex responder gene : conserved motifs and evolution with punctuated equilibria*, Human Molecular Genetics 1993. 2: 2075-2079 and Bibbins, K. B., Tsai, J. Y., Schimenti, J., Sarvetnick, N., Zoghbi, H. Y., Goodfellow, P. and Silver, L. M., *Human homologs of two testes-expressed loci on mouse chromosome 17 map to opposite arms of chromosome 6,* Genomics 1989. 5: 139-143) and mouse (Schimenti, J., Cebra-Thomas, J. A., Decker, C. L., Islam, S. D., Pilder, S. H. and Silver, L. M., *A candidate gene family for the mouse t complex responder (Tcr) locus responsible for haploid effects on sperm function*, Cell 1988. 55: 71-78; Ewulonu, U. K., Snyder, L., Silver, L. M. and Schimenti, J. C., *Promoter mapping of the mouse Tcp-10bt gene in transgenic mice identifies essential male germ cell regulatory sequences,* Molecular Reproduction and Development 1996. 43: 290-297 and Cebra-Thomas, J. A., Decker, C. L., Snyder, L. C., Pilder, S. H. and Silver, L. M., *Allele- and haploid-specific product generated by alternative splicing from a mouse t complex responder locus candidate*, Nature 1991. 349: 239-241) *TCP-10* is a T-complex responder (TCP) gene that may play a role in the transmission ratio distortion phenotype. A region of LAP is 56% identical to the 181 C-terminal residues of human TCP-10 protein and 66% identical to the 106 C-terminal residues of the murine TCP-10 protein.

The 5′ end of the LAP cDNA was further extended by 5′RACE cloning starting from PHA-blasts mRNA. Analysis of the LAP cDNA revealed a nucleotide sequence of 1353 bases that contains a single open reading frame (ORF) of 372 amino acids. This ORF starts at position 70 and ends with the translation stop codon, TGA, located at nt 1186.

It was found that this LAP sequence is in 99% identical (9 nt mismatches including 4 in the coding region with a single a.a. difference at the carboxy-terminus) to the 3′end of the recently published CPAP (centrosomal P4.1-associated protein) molecule, which is part of the γ-tubulin complex (Hung, L. Y., Tang, C. C. and Tang, T. K., *Protein 4.1 R-135 interacts with a novel centrosomal protein (CPAP) which is associated with the gamma-tubulin complex*, Mol. Cell. Biol. 2000. 20: 7813-7825). These 9 nt mismatches were also found on several EST sequences, confirming the differences observed between CPAP and LAP. (Hung, L. Y., et al. *Mol. Cell. Biol.* 2000. 20: 7813-7825).

Sequence identity of TCP-10, CPAP and LAP proteins is restricted to two conserved regions at the COOH-terminus. One carries a leucine zipper, which may form a series of heptads. Repeats involved in coiled-coil formations, and the second contains unusual glycine repeats (Hung, L. Y., et al. *Mol. Cell. Biol.* 2000. 20: 7813-7825).

Additional tests were performed in order to verify whether human LAP could bind to the murine LAG-3 IC region. It was observed a weak interaction between these two heterologous proteins with a small activation of the HIS3 gene but no detectable LacZ activity. Next, it was examined which region of human LAG-3 interacts with LAP. The binding of LAP with hLAG-3/IΔC and hLAG-3/EP constructs was tested in yeast cells and it was found that LAP indeed binds specifically with the short C-terminal region of LAG-

3 containing the EP-rich region. These results illustrating interaction of LAP and LAG-3 proteins (a) are shown in table 1 hereafter.

Table 1

fused to LexA BD			fused to Gal4 AD		
	LAG-3 regions	-	Lamin	RalB	LAP
hLAG-3/I	R457 to L503	-	-	-	++++
NLS-hLAG-3/I	R457 to L503	-	-	-	+++++
NLS-mLAG-3/I	L456 to L507	-	-	-	+
NLS-hLAG-3/I?C	R457 to E481	-	-	-	+/-
hLAG-3/EP	E478 to L503	-	-	-	++
NLS/hLAG-3/EP	E478 to L503	-	-	-	++++

Where LAG proteins are hLAG-3 and mLAG-3, IC regions were expressed as fusion proteins to the LexA DNA binding domain (LexA BD) in the pLex vector containing or not a nuclear localization sequence (NLS). The pGAD vector encoded the GAL4 activation domain (GAL4 AD) alone or fused to LAP or an unrelated protein (Lamin or RalB). Two procedures for interaction studies were performed: (i) co-transfection of yeast strain L40 with the two indicated plasmid combinations shown, (ii) transformation of strain L40 with a pLex construct which are then mated with strain AMR70 transformed with a pGAD construct.

A demonstration of *in vitro* binding between LAP and hLAG-3 proteins, LAP linked to GST or GST alone were expressed in bacteria and bound to glutathione-Sepharose beads was performed as described hereafter.

Bound proteins were incubated with total cell lysates prepared from PHA-activated T lymphocytes. The results demonstrate that the LAG-3 protein was specifically precipitated from the T-cell lysate when using affinity beads containing the LAP protein (Fig. 1A). The control GST beads did not precipitate any detectable LAG-3 protein from the T-cell lysate. Therefore, LAG-3 binds specifically to the LAP protein *in vitro*, in agreement with the data obtained from the yeast two-hybrid screening procedure.

In order to verify that the interaction between LAP and LAG-3 proteins in both the yeast two-hybrid system and in T-cell lysates does not require an additional adaptor protein, a direct binding assay in which the *in vitro*-translated LAG-3 protein was tested for interaction with beads bound to GST-LAP or GST alone was performed.

As shown in Figure 1B, affinity beads containing the GST-LAP fusion protein pulled down the LAG-3 protein in a specific manner. This supports the existence of a specific direct physical interaction between LAP and LAG-3 proteins without the need for the presence of a third adaptor protein.

Overall, the interaction between LAP and hLAG-3 has been confirmed both *in vivo* and *in vitro* using recombinant LAP protein. In particular, it was showed that the LAP protein was able to bind LAG-3 in lysates of activated T cells. This interaction was specific and was also observed vice versa using *in vitro* translated recombinant LAG-3.

2.3 The C-terminus region of LAP binds the EP region of hLAG-3.

To determine the region of the LAP protein that contains the LAG-3 binding site, deletion mutants of the LAP cDNA was constructed (Fig. 2A). The binding of these mutants with hLAG-3/I, hLAG-3/IΔC and hLAG-3/EP were tested, with Ral B as a negative control. Deletion of the extreme C-terminal regions (mutant D3) already abolished some binding activity (Fig. 2C), while the shorter constructs (D1 & D2) did not bind to hLAG-3 at all.

Thus, the binding site for LAP on the EP motifs is located in its C-terminal region.

LAP would then function to cluster rafts into the immunological synapse following TCR engagement, a phenomenon that requires the polarization of actin and microtubules (Simons, K. and Toomre, D., Lipid rafts and signal transduction Nature 2000. 1: 31-39).

2.3 LAP binds to the intracytoplasmic region of the PDGF receptor containing an EP motif

The PDGF receptor (Claesson-welsh, L., A.Eriksson, A.Morén, L.Severinsson, B.Ek, A.Ostman, C.Betsholtz and C.H.Heldin, *cDNA cloning and expression of a human platelet-derived growth factor (PDGF) receptor specific for B-chain-containing PDGF molecules,* Mol. Cell. Biol. 1988. 8: 3476-3486) has a long intracytoplasmic tail containing numerous motifs known to be involved in signalling. It was noticed that a repeated EP motif not known to be involved in transduction signalling was found in its C-terminal region (Figure 2B).

Surprisingly the LAP protein could bind to this EP motif-containing segment.

Thus, LAP interactions with other membrane receptor intracytoplasmic regions containing the EP motif have crearly been identified, since the present work shows that it binds to the PDGFR intracellular region in addition to hLAG-3 and mLAG-3.

Thus, this EP motif appears as a common transduction motif, that could be used by other functionally important receptors.

2.4 LAP is a 45 kDa protein expressed in all tested human cells

To determine the size and expression of the LAP protein, total cell lysates were analyzed by Western blotting with a rabbit polyclonal serum rose against a LAP peptide with no sequence homology with TCP-10. Two bands at 30- and 45 kDa were detected in PBMCs on activated T-cells (Figure 3). The 30 kDa band was shown to be non-specific since it was also detected using the preimmune serum (Fig. 3). The 45 kDa band corresponds to LAP as it was no longer detected following pre-incubation of the immune serum containing the LAP peptide (10^{-6} M at 4°C for 1 hr) (Figure 3) while pre-incubation with a control peptide had no effect (data not shown). In addition, this 45 kDa band was found in cytoplasmic but not in nucleic T cell extracts (data not shown).

These results clearly indicate that LAP is expressed as a 45 kDa cytoplasmic protein in PBMCs and in activated T cells with a higher expression level in the latter cells.

Western blotting was also performed with total cell lysates of the Jurkat T cell line, two EBV-transformed B cell lines and a renal cell carcinoma cell line (RCC7).

LAP is also expressed in these cell lines as a 45 kDa protein with lower expression in PBMC (data not shown). LAP is thus expressed in T and non-T hematopoietic cell lines as well as in non-hematopoietic cell lines.

In addition, LAP was detected in different untransformed human tissues, including the lung, liver, kidney, testes (no overexpression, in contrast to CPAP), pancreas and heart, but not in the spleen and brain (data not shown).

2.5 Two RNA species are derived from the LAP gene.

The LAP gene was first analysed by digesting DNA from different cell lines and PBLs, Southern blotting and hybridizing using the LAP cDNA as a probe. Unique EcoRI (5.5 kb), Hind III (9 kb) and Xho I (>12 kb) fragments were found indicating that the LAP or CPAP gene is either present in the human genome as a single copy gene or represents two closely related genes (data not shown).

Total and poly-A^+ RNA samples of PHA-blasts were run on a denaturing agarose gel and analyzed by Northern blotting. The LAP RNA seemed to be rarely expressed, as it was only detected by using 15 µg of poly-A^+ RNA while not being detected in total RNA samples (up to 20 µg, data not shown). Two faint bands hybridised with the labelled cDNA LAP probe, one with a size of 4.5 kb and a weaker one at 1.8 kb. As these two bands correspond exactly to the sizes of the 28S and 18S rRNA, the blot was then rehybridized with saturating amounts of ribosomal RNA (10 µg/ml) added to prevent any non-specific binding of the probe to the remaining rRNA in the sample and the same result was obtained (data not shown). Since these two signals were only seen with highly purified poly-A^+ RNA and

not with total RNA samples containing a greater amount of rRNA, we concluded that LAP was specifically expressed as a 1.8 kb mRNA. The stronger 4.5 kb signal may correspond to CPAP, which has been shown to be weakly expressed in most tissues, except testis (Hung, L. Y., Tang, C. C. and Tang, T. K., *Protein 4.1 R-135 interacts with a novel centrosomal protein (CPAP) which is associated with the gamma-tubulin complex,* Mol. Cell. Biol. 2000. 20: 7813-7825).

Thus, LAP is a new human protein, expressed in all tested human cells and derived from a rare mRNA. It appears that LAP and CPAP are derived from either a single gene or two closely related genes which are strongly expressed in the testes for the CPAP mRNA (4.5 kb) and weakly expressed in other cells as two messages (4.5 kb and 1.8 kb) coding for CPAP and LAP, respectively.

The specific immunoprecipitation of LAG-3 by LAP-GST beads from activated T lymphocyte lysates indicates that the two overlapping 150 kDa CPAP (Hung, L. Y., Tang, C. C. and Tang, T. K., *Protein 4.1 R-135 interacts with a novel centrosomal protein (CPAP) which is associated with the gamma-tubulin complex*, Mol. Cell. Biol. 2000. 20: 7813-7825) and 45 kDa LAP proteins have different functions, that is binding to γ-tubulin in the centrosome especially in testis cells (CPAP) or binding to EP motifs present on membrane expressed receptors (LAP).

EP motifs are rare in human proteins, and the specific binding of LAP on such motifs has important biological significance for signal transduction and/or coupling of clustered rafts to the microtubule networks.

3 Materials and methods

3.1 Plasmid construction

The hLAG-3/I and mLAG-3/I fragments encode the full length intracellular region of human LAG-3 and murine LAG-3, respectively. The hLAG-3/IΔC encodes the intracellular domain of human LAG-3 deleted of its 22 C-terminal amino acids (ΔC) whereas hLAG-3/EP codes only for the EP-rich region located at the end of the C-terminal part of hLAG-3. The PCR products were cloned into the two hybrid vectors pBMT116 (pLex) or a derivative containing an additional Nuclear Localization Sequence (pLex/NLS) (Vojtek, A. B. and Hollenberg, S. M., *Ras-Raf interaction : two-hybrid analysis,* Methods Enzymol. 1995. 255: 331-342) in frame with the LexA DNA binding protein yielding the following constructs:

- pLex-hLAG-3/I and pLex/NLS-hLAG-3/I (from R^{457} to L^{503})
- pLex/NLS-mLAG-3 (from L^{456} to L^{507})
- pLex-hLAG-3/IΔC and pLex/NLS-hLAG-3/IΔC (from R^{457} to E^{481})
- pLex-hLAG-3/EP and pLex/NLS-hLAG-3/EP (from E^{478} to L^{503}).

3.2 Two-hybrid screen and interaction analysis

Yeast, medium and two-hybrid procedures were handled according to published methods (Vojtek, A. B. and Hollenberg, S. M., *Ras-Raf interaction : two-hybrid analysis*, Methods Enzymol. 1995. 255: 331-342; Kaiser, C., Michaelis, S. and Mitchell, A., Methods in yeast genetics Cold Spring Harbor Laboratory 1994). For the two hybrid-screen, it was used a human activated PBL library cloned in the pGAD-1318 vector (Hybrigenics, Paris, France) which contains the activation domain of GAL4 under the control of the entire ADH1 strong yeast promoter. For library

screening, yeast strain L40 which contains the LacZ and HIS3 reporter genes downstream of the binding sequence of LexA, was sequentially transformed with pLex/NLS-hLAG-3/I and 60 µg of the human activated T cell library using the lithium acetate method. Double transformants were plated on yeast drop-out medium lacking tryptophan, leucine and histidine, and were incubated at 30°C for 3 days. Positive colonies His^+ were patched on selective plates for growth and were then replicated on Whatman 40 paper. The β-galactosidase activity was tested by a filter assay.

For interaction studies, two methods were used : by co-transformation of strain L40 with pairs of pLex and pGAD vectors, or by mating the strain L40 expressing a pLex vector with the strain AMR70 containing a pGAD vector. In both cases, binding was tested for growth in histidine-deficient medium and for β-galactosidase activity. Signals described as being negative were not detected even after 3 days or 24 hrs for the HIS3 and LacZ reporter genes, respectively. No discrepancy was ever observed between the histidine auxotrophy and the β-galactosidase tests.

3.3 Protein expression and purification

LAP polypeptide was expressed as a glutathione S-transferase (GST) fusion protein in *Escherichia coli* and immobilized on affinity matrix beads. Briefly, fresh overnight cultures of *E. Coli* HB101 or XL-1 blue cells harboring the pGEX plasmid expressing GST or GST-LAP proteins were diluted 1 :10 in Luria-Bertani (LB) broth supplemented with 20 µg/ml ampicillin and the cultures were grown for 3 h with 0.1 mM IPTG (Sigma, St. Louis, MO). Cell pellets were collected by centrifugation and lysed in Tris buffer containing 1% NP-40 and anti-proteases. The soluble

fraction was prepared by centrifugation at 10,000 g for 15 min at 4°C. The GST and recombinant GST fusion proteins were purified by coupling to Glutathione Sepharose 4B beads (Pharmacia, Uppsala, Sweden) by gentle mixing at 4°C for 40 min followed by extensive washing. The protein-bound affinity beads were analyzed and quantitated by Coomassie blue R-250 staining following SDS-PAGE analysis.

3.4 Preparation of cell lysates and in vitro binding assays

Human PBMCs were isolated from venous blood by Ficoll-Paque density gradient centrifugation. T lymphocytes were obtained by stimulating PBMCs with 1 μg/ml of PHA-P (Wellcome, Beckenham, UK) at 37°C and 10% CO_2 in complete culture medium (RPMI 1640 supplemented with 10% heat inactivated human AB serum, 4 mM L-glutamine, 1 mM pyruvate, 0.2 mM NaOH, 50,000 IU penicillin and 50 mg/ml streptomycin). After 3 days of culture, whole cell lysates were prepared in Tris cell lysis buffer containing 1% NP-40 and anti-proteases.

The hLAG-3 protein was synthesized *in vitro* using the T7-coupled rabbit reticulocyte lysate system (TNT, Promega, Madison, WI). Equal amounts of GST-LAP or control GST proteins immobilized on beads were incubated for 3 hrs at 4°C with direct whole cell lysates (after centrifugation of nuclei) or with the *in vitro* translated hLAG-3 protein in a binding buffer (20 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin). Bound proteins were then extensively washed in PBS buffer and analyzed by Western blotting.

3.5 Cell lines and antibodies

The Jurkat T cell line and the Epstein Barr Virus (EBV)-transformed B cell line were grown in complete 1640 RPMI culture medium at 37°C and 6 % CO_2. RCC7 (a renal cell carcinoma cell line, (Gaudin, C., Kremer, F., Angevin, E., Scott, V. and Triebel, F., *A HSP70-2 mutation recognized by cytolytic T lymphocytes on a human renal cell carcinoma*, J. Immunol. 1999. 162: 1730-1738) were cultivated in complete DMEM medium at 37°C and 6 % CO_2.

A polyclonal serum was raised against a peptide (SPREPLEPLNFPDPEYK) derived from the deduced amino-acid sequence of LAP by immunizing rabbits with three injections of peptide-BSA (Neosystem, Strasbourg, France).

3.6 Western blot

10^6 cells were washed and lysed at 4°C for 60 min in 100 µl Tris cell lysis buffer. Cell debris were removed by 10 min centrifugation at 10,000 g and the lysates heat-denaturated in SDS sample buffer for 5 min. Total cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were saturated with 5% dry milk for 1 hr at 37°C and incubated with primary antibody diluted 1:3000 in TBS for 1.5 hr with slow agitation. After incubating the membranes with the GAR-peroxidase secondary antibody, the signal was detected by enhanced chemiluminescence (ECL, Amersham, Buckinghamshire, UK). To determine the tissue distribution of LAP, a commercial Western blot containing 75 µg of total cellular protein from eight different human tissues (Chemicon, Temecula, USA) was used.

CLAIMS

1) A molecule binding to a target comprising an EP motif having the following sequence :

$[X-(EP)_n-Y-(EP)_m-Z]_p$

wherein X, Y and Z, identical or different comprise a sequence of 0 to 10 aminoacids, identical or different, n, m are integers comprised between 0 to 20, preferably between 3 to 10 at least one from n or m being different from 0 and p is an integer comprised between 1 and 10.

2) A molecule according to claim 1 which binds to an EP motif selected from the group comprising the following formula: EPEPEPEPEPEPEPEPEP (SEQ ID N° 3), EPEPEPQLEPEP (SEQ ID N° 4), EPQDEPPEPQLELQVEPEPELEQ (SEQ ID N° 5), or EPEPEPEPEPEP (SEQ ID N° 6).

3) A molecule according to claim 1 which binds to an aminoacid sequence comprising at least 5 EP motifs over a 19 aminoacid segment.

4) A molecule according to anyone claims 1 to 3 wherein the molecule is selected from a peptide, a polypetide or a protein.

5) A polypeptide according to claim 4 consisting of or comprising the amino acids sequence of LAP identified by SEQ ID No.:1 of the sequence listing in annexe, an homologous, a fragment or a derivative thereof.

6) A polypeptide according to claim 4 consisting of or comprising the carboxy-terminal amino acids sequence of LAP identified by SEQ ID No.:2 of the

sequence listing in annexe, an homologous, a fragment or a derivative thereof.

7) A nucleic acid molecule consisting of or comprising a polynucleotide sequence coding a polypeptide according to claim 4.

8) A nucleic acid molecule according to claim 7, consisting of or comprising the polynucleotide sequence identified by SEQ ID No.:8 a fragment or a derivative thereof.

9) An expression vector comprising a nucleic acid molecule according to claims 7 or 8.

10) A host cell transformed with an expression vector according to claim 9.

11) Process for the manufacturing of a molecule according to claim 4, comprising :

a)the transfection of a host cell with an expression vector according to claim 5 to obtain the expression of the polypeptide,

b)the isolation and purification of the polypeptide from the transfected host cell.

12) A pharmaceutical composition comprising as active agent at least one molecule according to claims 1 to 6.

13) A pharmaceutical composition according to claim 12 useful for treating immune-related pathologies.

14) A pharmaceutical composition according to claim 12 useful for modulating the immune response .

15) A pharmaceutical composition according to claim 12 useful to enhance the development of CD4 or CD8 T-cell populations.

16) A pharmaceutical composition according to claim 12 useful to suppress the development of CD4 or CD8 T-cell populations.

17) A pharmaceutical composition according to anyone of claims 12 to 16 wherein said molecule is a LAP agonist.

18) A pharmaceutical composition according to anyone of claims 12 to 16 wherein said molecule is a LAP antagonist.

19) Use of a molecule according to claims 1 to 6 for the manufacture of a pharmaceutical composition useful for treating immune-related pathologies.

20) Use of a molecule according to claims 1 to 6 for the manufacture of an pharmaceutical composition useful for immunomodulate the immune response

21) Use of a molecule according to claims 1 to 6 for the manufacture of a therapeutical composition enhancing the development of CD4 or CD8 T-cell populations.

22) Use of a molecule according to claims 1 to 6 for the manufacture of a therapeutical composition suppressing the development of CD4 or CD8 T-cell populations

23) Use according to anyone of claims 19 to 22 wherein said molecule is a LAP agonist

24) Use according to anyone of claims 19 to 22 wherein said molecule is a LAP antagonist

25) A method for screening drugs comprising the steps of :

-put in contact the candidate drug with a molecule according to claims 1 to 6 in the presence of its target EP motif,

-measure the resulting binding of said molecule to its target.

26) A method according to claim 25 wherein said drugs are selected from drugs able to activate T-cell, drugs enhancing the development of CD4 or CD8 T-cell populations, drugs suppressing the development of CD4 or CD8 T-cell populations, drugs active in platelet activation

27) A method according to claims 25 or 26 wherein the molecule according to claims 1 to 5 is a LAP polypeptide.

28) Antibodies directed to a specific epitope of a polypeptide selected from the group consisting of polypeptides or peptides identified by SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:9, from the sequence listing on annexe.

29) Antibodies according to claim 28 wherein said antibodies are monoclonal antibodies or Fab, Fab', F(ab') or Fv fragments thereof.

30) A monoclonal antibody or a monoclonal antibody derivative which specifically binds a peptide selected from the group consisting of polypeptides or peptides identified by SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6 or SEQ ID NO:9, from the sequence listing on annexe, said monoclonal antibody derivative being selected from the group consisting of a monoclonal antibody conjugated to a cytotoxic agent or a radioisotope, and Fab, Fab' or $F(ab')_2$ fragments of said monoclonal antibody conjugated to a cytotoxic agent or radioisotope.

31) A hybridoma cell line producing the monoclonal antibody of claim 30.

32) A therapeutic composition comprising as active ingredient an antibody according to claims 28 to 30.

33) Use of antibodies according to claims 28 to 30 in a method for purifying, identifying or quantified a polypeptide according to claims 4 to 6 or its homologous.

34) Use of antibodies according to claims 28 to 30 to screen drugs.

ABSTRACT

The present invention relates to molecules binding to specific targets comprising Glu-Pro (EP) repeated motifs, namely to molecules binding to a target comprising an EP motif having the following sequence $[X-(EP)_n-Y-(EP)_m-Z]_p$ wherein X, Y and Z, identical or different comprise a sequence of 0 to 10 aminoacids, identical or different, n, m are integers comprised between 0 to 20, preferably between 3 to 10 at least one from n or m being different from 0 and p is an integer comprised between 1 and 10. The invention relates also to pharmaceutical compositions containing them, to antibodies directed against them and to methods useful for screening drugs by using them.

Figure 1A

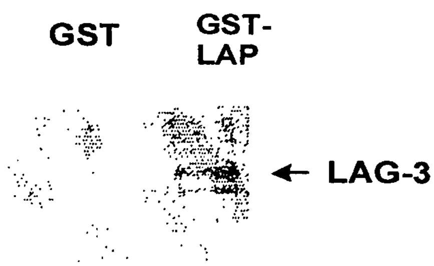

Figure 1 B

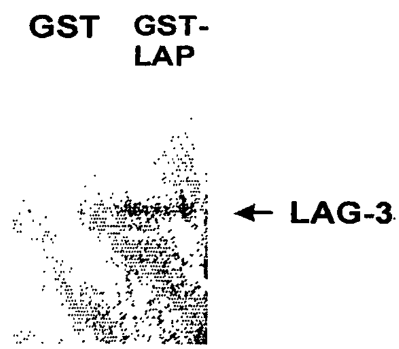

Figure 2 A

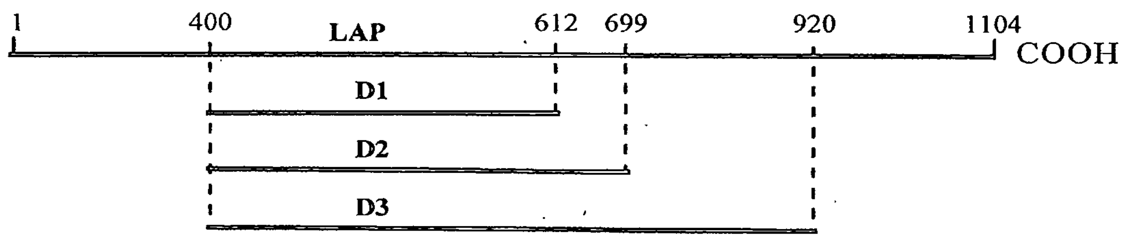

Figure 2 B

PDGFR/EP :
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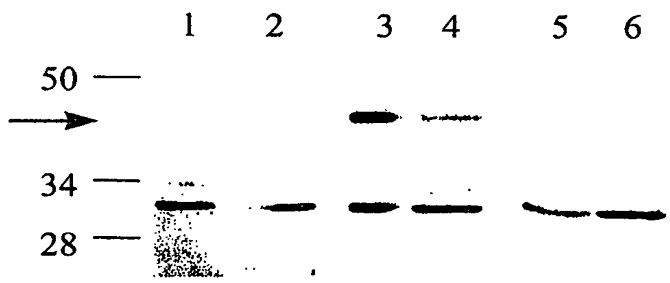
DTSSVLYTVQPNEGDNDYIIPLPDPKPEVADEGPLEGSPSLASSTLNEVNTSS
TISCDSPLEPQDEPPEPQLELQVEPEPELEQLPDSGCPAPRAEAEDSFL 1106

Figure 2 C

fused to LexA BD	fused to Gal4 AD LAP	D3	D2	D1
hLAG-3/I	++++	++	-	-
hLAG-3/ IΔC	+/-	-	-	-
hLAG-3/EP	+++	-	-	-
RalB	-	-	-	-
PDGFR/EP	++++	-	-	-

FIGURE 3



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Université d'Orsay Paris-Sud

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